Actin Assembly in Electropermeabilized Neutrophils: Role of Intracellular Calcium

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Abstract. Assembly of microfilaments involves the conversion of actin from the monomeric (G) to the filamentous (F) form. The exact sequence of events responsible for this conversion is yet to be defined and, in particular, the role of calcium remains unclear. Intact and electropermeabilized human neutrophils were used to assess more directly the role of cytosolic calcium ($[Ca^{2+}]_i$) in actin assembly. Staining with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin and right angle light scattering were used to monitor the formation of F-actin. Though addition of Ca^{2+} ionophores can be shown to induce actin assembly, the following observations suggest that an increased $[Ca^{2+}]_i$ is not directly responsible for receptor-induced actin polymerization: (*a*) intact cells in Ca^{2+}-free medium, depleted of internal Ca^{2+} by addition of ionophore, responded to the formyl peptide fMLP with actin assembly despite the absence of changes in $[Ca^{2+}]_i$, assessed with Indo-1; (*b*) fMLP induced a significant increase in F-actin content in permeabilized cells equilibrated with medium containing 0.1 μM free Ca^{2+}, buffered with up to 10 mM EGTA; (*c*) increasing $[Ca^{2+}]_i$ beyond the resting level by direct addition of $CaCl_2$ to permeabilized cells resulted in actin disassembly. Conversely, lowering $[Ca^{2+}]_i$ resulted in spontaneous actin assembly. To reconcile these findings with the actin-polymerizing effects of Ca^{2+} ionophores, we investigated whether A23187 and ionomycin induced actin assembly by a mechanism independent of, or secondary to the increase in $[Ca^{2+}]_i$. We found that the ionophore-induced actin assembly was completely inhibited by the leukotriene B_4 (LTB_4) antagonist LY-223982, implying that the ionophore effect was secondary to LTB_4 formation, possibly by stimulation of phospholipase A_2. We conclude that actin assembly is not mediated by an increase in $[Ca^{2+}]_i$, but rather that elevated $[Ca^{2+}]_i$ facilitates actin disassembly, an effect possibly mediated by Ca^{2+}-sensitive actin filament-severing proteins such as gelsolin. Sequential actin assembly and disassembly may be necessary for functions such as chemotaxis.

DESPITE extensive study, the mechanisms responsible for initiating the conversion of G- to F-actin (polymerization, assembly) after receptor-mediated stimulation in neutrophils are still incompletely understood (see reviews by Korn, 1982; Naccache, 1987; Omann et al., 1987; Sha'afi and Molski, 1988). There is evidence pointing to the involvement of GTP-binding regulatory proteins (G proteins): the actin polymerization response induced by most soluble activators is blocked by pretreatment of the cells with pertussis toxin (Shefeyk et al., 1985). Moreover, in permeabilized cells actin assembly can be induced in the absence of stimuli by addition of GTP-γ-S or fluoroaluminate (AlF_4^-) and these responses, as well as those mediated by receptors, are inhibited by GDP-β-S (Downey et al., 1989; Therrien and Naccache, 1989). By comparison, the nature of the events that occur following activation of the G protein(s) remains unclear. There is evidence implicating the interaction of membrane phosphoinositides with actin-binding and sequestering proteins such as profilin (Lassing and Lindberg, 1985; Stossel, 1989) and other data suggest involvement of protein kinase C (reviewed by Omann et al., 1987, and Sha'afi and Molski, 1988), but the precise mechanisms involved have not been defined.

Calcium plays a central role in signal transduction for many responses in neutrophils (Sha'afi and Molski, 1988). An early increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$)[1] has been recorded after stimulation of neutrophils with a variety of agents (Bengtsson et al., 1986; Pozzan et al., 1983), leading to the suggestion that this calcium "transient" might signal the initiation of actin polymerization (Stendahl and Stossel, 1980). Evidence favoring this hypothesis includes: (*a*) the ability of calcium ionophores to promote actin assembly (Howard and Wang, 1987); (*b*) the observation that most neutrophil agonists that cause actin polymerization also induce an increase in intracellular Ca^{2+} (see review by Lew,

1. *Abbreviations used in this paper*: Ca^{2+}, intracellular calcium; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LTB_4, leukotriene B_4; PAF, platelet activating factor; RFI, relative fluorescence index.

1989); (*c*) the similarity in kinetics and dose response relationships of the increase in $[Ca^{2+}]_i$ and actin polymerization induced by physiological stimuli (Naccache, 1987); and (*d*) the observation that actin polymerization induced by adherence to plastic surfaces was inhibited in a calcium-free medium (Southwick et al., 1989). On the other hand, other observations suggest that changes in $[Ca^{2+}]_i$ are neither necessary nor sufficient for actin polymerization to occur. The latter conclusion was derived from observations that (*a*) agents such as PMA can induce significant actin assembly without a detectable increase in $[Ca^{2+}]_i$ (Sheterline et al., 1986) and (*b*) inhibition of the $[Ca^{2+}]_i$ transients with quinacrine or by loading the cytosol with calcium buffering agents failed to inhibit *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induced actin assembly (Sha'afi et al., 1986). It could be argued, however, that local $[Ca^{2+}]_i$ increases persisted under these conditions, which escaped detection by the methods used.

Clearly, the relationship between the increase in $[Ca^{2+}]_i$ and actin polymerization remains controversial. In this report, intact and permeabilized human neutrophils were used to study this relationship in more detail. Electropermeabilized cells can be equilibrated with EGTA-buffered media of defined $[Ca^{2+}]$, while retaining responsiveness to physiological ligands, such as chemoattractants (Grinstein and Furuya, 1986; Downey and Grinstein, 1989). This experimental model enabled us to test directly the effects of varying $[Ca^{2+}]_i$ on actin assembly, in the absence of activators or ionophores. Conversely, we were also able to analyze the stimulation by fMLP under conditions where the change in intracellular calcium was effectively prevented.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia Fine Chemicals (Montreal, Canada). Reagents for Krebs Ringers phosphate dextrose buffer (KRPD) were obtained from Mallinckrodt Inc. (Paris, KY). EGTA, fMLP, A23187, Hepes and ATP (K^+ salt) were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-phallacidin, acetoxymethyl ester of Indo-1 (Indo 1-AM), and BCECF-AM were obtained from Molecular Probes (Eugene, OR). Lysophosphatidylcholine (lyso PC) was obtained from Avanti Polar Lipids (Pelham, AL), and ionomycin and nigericin were obtained from Calbiochem-Behring Corp. (San Diego, CA). WEB2086 and L659,989 were a kind gift from Dr. P. Hellewell, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

Cell Isolation

Human neutrophils (>98% pure) were isolated from citrated whole blood obtained by venipuncture using dextran sedimentation and discontinuous plasma-Percoll gradients as previously described in detail (Haslett et al., 1985). The separation procedure required 2 h and the cells were used immediately for the experiments described. The functional integrity and nonactivated state of neutrophils isolated in this manner has been extensively validated in previous publications (Downey et al., 1988; Worthen et al., 1989).

Cytosolic Calcium and Light Scattering Determinations

Intracellular free calcium concentration and right angle light scattering were determined using Hitachi F2000 and F4000 fluorescence spectrometers. For the measurement of $[Ca^{2+}]_i$, the cells were loaded with Indo-1 by preincubation with 1.5 μg/ml of the precursor acetoxymethylester at 37°C for 30 min as described (Nasmith and Grinstein, 1987). Aliquots of this cell suspension were washed by sedimenting in a microcentrifuge and resuspending in KRPD buffer and then placed in the indicated medium in a plastic cuvette with magnetic stirring. Fluorescence was measured with excitation at 331 nm and emission at 410 nm, using 5- or 10-nm slit widths. $[Ca^{2+}]_i$ was calibrated using ionomycin and Mn^{2+} as described (Nasmith and Grinstein, 1987). Right angle light scatter was monitored at either 340 or 550 nm, as described by Sklar et al. (1985). Similar results were obtained at both wavelengths. The data are expressed as percent of the initial scattering of untreated cells.

Permeabilization Procedure

Neutrophils were permeabilized by electroporation essentially as described (Grinstein and Fuyura, 1988). Briefly, 10^7 cells were sedimented and resuspended in 1 ml of ice-cold permeabilization medium (140 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM ATP, 10 mM Hepes, pH 7.0, 1–10 mM EGTA, as indicated, and the appropriate amount of $CaCl_2$ to give the final free Ca^{2+} concentration specified in the text, calculated by the method of Fabiato and Fabiato [1979]. Aliquots of this suspension (0.8 ml) were transferred to a cuvette (Pulser; Bio-Rad Laboratories, Cambridge, MA) and subjected to two discharges of 2 kV from a 25 μF capacitor using the gene pulser (Bio-Rad Laboratories). The cells were sedimented and resuspended in fresh ice-cold medium between pulses. Finally, the cells were equilibrated for 30 s in the indicated medium at 37°C before stimulation and measurement of actin polymerization. In the set of experiments described in Fig. 3, neutrophils were permeabilized in medium with 100 nM $[Ca^{2+}]_{free}$, allowed to equilibrate for 1 min, and then the appropriate amount of $CaCl_2$ added to give the final $[Ca^{2+}]_{free}$ specified in the text. In the set of experiments described in Fig. 5, neutrophils were permeabilized in medium with the specified $[Ca^{2+}]_{free}$, allowed to equilibrate for 1 minute and then exposed to fMLP 10^{-8} M for an additional minute.

Flow Cytometry

Neutrophil content of polymerized actin (F-actin) was determined by NBD-phallacidin staining of fixed and permeabilized cells (Howard and Meyer, 1984), as previously described (Downey et al., 1989). This fluorescence method has been shown to correlate well with biochemical measurements of F-actin (Howard and Meyer, 1984; Wallace et al., 1984). The stained cells were analyzed on an Epics 5 or an Epics Profile fluorescence-activated cell sorter (Coulter Electronics Inc., Hialeah, FL). Cells were excited with an argon laser at 488 nm and emission recorded at 520 nm with band pass and short pass filters. Gating was done on the forward angle and right angle light scatter only to exclude debris and cell clumps. A minimum of 10,000 cells were measured per condition and all values are expressed as relative fluorescence index (RFI). For experiments carried out on the Epics 5, the RFI was calculated according to the formula $RFI = 2[(b - a)/26]$, where a equals mean channel number of the control cell population, b equals mean channel number of the cell population in question, and 26 equals number of channels representing a doubling of fluorescence intensity. For experiments carried out on the Coulter Profile, the RFI was calculated using the ratio of the linearized mean fluorescence of the cell populations in question, as provided by the data processing software.

Statistical Analysis

Data are reported as mean ± SEM of the number of experiments indicated. All data was analyzed by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe), except the data in Fig. 2 which were analyzed by the paired t test.

Results and Discussion

Is Increased $[Ca^{2+}]_i$ Required for Actin Polymerization?

Fig. 1 illustrates a comparison of the kinetics of the increase in cytosolic free calcium ($[Ca^{2+}]_i$) and of the change in right angle light scatter, used here as an index of actin polymerization (see Sklar et al., 1984). In agreement with earlier observations (Yuli and Snyderman, 1984; Sklar et al., 1984), the addition of fMLP to intact cells was followed almost immediately by a decrease in light scattering (Fig. 1 *a*, *bottom*

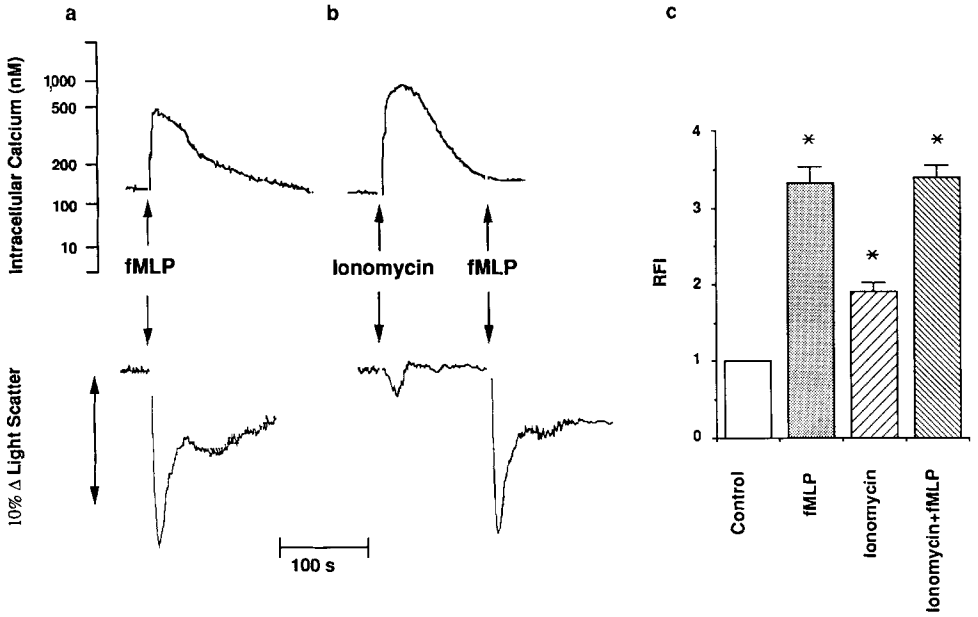

Figure 1. Relative time courses of intracellular calcium (*a* and *b*, *top traces*) and right angle light scatter (*a* and *b*, *bottom traces*) in neutrophils in response to stimulation with 10^{-8} M fMLP or 10^{-6} M ionomycin in low calcium medium (<10 nM). The arrow indicates the time at which the specified stimulus was added. The discontinuity of the traces represents the artifact created by opening the lid of the sample compartment of the fluorimeter that closes the shutter of the photomultiplier tube. Each trace is representative of at least four determinations. Note the failure of fMLP to increase intracellular calcium after pretreatment with ionomycin in low calcium medium (*b*, *top trace*) but that the changes in light scattering under the same conditions remain intact (*b*, *bottom trace*). (*c*) Changes in F-actin content of neutrophils in response to stimulation with 10^{-8} M fMLP, 10^{-6} M ionomycin, or pretreatment with 10^{-6} M ionomycin followed by stimulation with 10^{-8} M fMLP in low calcium (<10 nM) medium. F-actin content is expressed as the RFI relative to control (untreated) cells, calculated as described in Materials and Methods. Each value represents the mean ± SEM of five determinations. Asterisks indicate $P < 0.05$ with respect to the control, determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).

trace) and a coincident increase in $[Ca^{2+}]_i$ (Fig. 1 *a*, *top trace*). At 37°C, the change in scattering peaked at 15–30 s and was followed by a more sustained, smaller decrease lasting several minutes. In intact cells treated with ionomycin in the presence (not shown) or absence of external Ca^{2+} (Fig. 1 *b*, *top trace*), there was a similar immediate increase in $[Ca^{2+}]_i$. However, in contrast with the rapid effect of fMLP, there was a discernible (1–3 s) lag before the onset of the light scattering change (e.g., Fig. 1 *b*, *bottom trace*). Because similar or larger $[Ca^{2+}]_i$ levels were attained with ionomycin than with fMLP, this temporal dissociation between the two phenomena suggests that increased $[Ca^{2+}]_i$ cannot by itself account for the rapid scattering response to fMLP, suggesting the existence of additional signals. Indeed, in cells pretreated with ionomycin in low $[Ca^{2+}]$ medium to deplete intracellular Ca^{2+} stores, subsequent exposure to fMLP did not result in an increase in $[Ca^{2+}]_i$ (Fig. 1 *b*, *top trace*), but the change in light scattering persisted (Fig. 1 *b*, *bottom trace*). As has been reported by others (Sklar et al., 1985), the return to baseline of the second (delayed) phase of actin polymerization was slowed in the cells depleted of calcium (compare the bottom traces in Figs. 1, *a* and *b*), suggesting a role for calcium in this phase.

NBD-phallacidin staining and flow cytometry were used to ascertain that the light scatter determinations accurately reflected changes in F-actin content. Pretreatment of the cells with ionomycin in low $[Ca^{2+}]$ medium, which produced a small decrease in light scattering (Fig. 1 *b*, *bottom trace*), resulted in a small but significant increase in F-actin content (Fig. 1 *c*). Subsequent exposure to fMLP resulted in further actin assembly (Fig. 1 *c*), even though $[Ca^{2+}]_i$ was not increased by the chemoattractant under these conditions. In view of these observations, signals other than changes in $[Ca^{2+}]_i$ must be invoked to account for the chemoattractant-induced initiation of actin polymerization.

Evidence against a requirement for an increased $[Ca^{2+}]_i$ for actin polymerization was also obtained using electrically permeabilized neutrophils. Such cells were earlier shown to allow the rapid equilibration of solutes of $M_r \leqslant 700$, while retaining the ability to respond to fMLP (Grinstein and Fuyura, 1986; Downey and Grinstein, 1989). In unstimulated cells, the permeabilization procedure itself caused a slight, variable increase in the content of F-actin by a mechanism that is presently not clear. Nevertheless, subsequent stimulation with 10^{-8} M fMLP for 1 min resulted in a further large increase in actin polymerization (Fig. 2). The

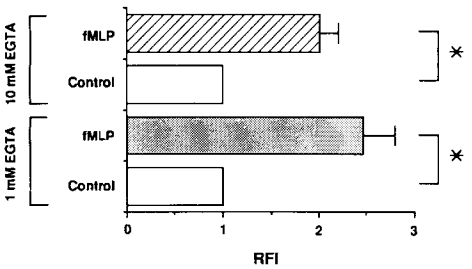

Figure 2. F-actin content of electropermeabilized neutrophils equilibrated with media containing 100 nM free calcium, buffered with either 1 or 10 mM EGTA as indicated. Where specified, the cells were stimulated with 10^{-8} M fMLP for 1 min at 37°C. Data are expressed as the RFI relative to control neutrophils incubated in a comparable concentration of EGTA. Each value represents the mean ± SEM of five determinations. Asterisks indicate $P < 0.05$ with respect to the control, determined by student's *t* test for paired data.

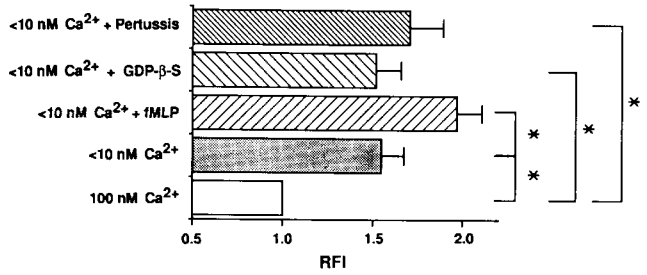

Figure 3. F-actin content of electropermeabilized neutrophils equilibrated with media containing 1 mM EGTA and either 100 nM or <10 nM free calcium (obtained by adding EGTA to nominally calcium free solution) as specified. Where indicated, the cells were treated with 10^{-8} M fMLP or 200 μM GDP-β-S, or pretreated with 500 ng/ml pertussis toxin for 2 h at 37°C. Data are expressed as the RFI relative to control neutrophils that are permeabilized and maintained in 100 nM free calcium. Each value represents the mean ± SEM of at least five determinations. Asterisks indicate $P < 0.05$ with respect to the control (100 nM free calcium), determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).

chemoattractant-stimulated polymerization was evident not only in the regular permeabilization medium, which is buffered at 100 nM [Ca^{2+}] with 1 mM EGTA, but also when the cytosol was equilibrated with a more heavily buffered solution, containing 10 mM EGTA but the same free [Ca^{2+}] (Fig. 2). The continuity between the external medium and the cytosol, together with the high buffering capacity of the medium used, ensured that changes in [Ca^{2+}]$_i$ in response to fMLP, if present, were minimal. Together, these observations indicate that an elevation of [Ca^{2+}]$_i$ is neither sufficient nor necessary for chemoattractant-induced actin assembly.

Effects of Varying [Ca^{2+}]$_i$ on Resting and Stimulated Actin Polymerization

Though an increase in [Ca^{2+}]$_i$ does not appear to be essential for actin polymerization, the presence of physiological (resting) [Ca^{2+}]$_i$ levels may have a "permissive" effect, as has been shown for the chemoattractant-induced respiratory burst (Sklar and Oades, 1985). To determine if the presence of intracellular calcium was required for actin assembly to occur, [Ca^{2+}]$_i$ was lowered to <10 nM by incubating electropermeabilized neutrophils with nominally calcium-free permeabilization buffer (5 mM EGTA with no added $CaCl_2$). As shown in Fig. 3, these conditions resulted in a spontaneous assembly of actin in the absence of exogenously added stimuli (RFI at 100 nM [Ca^{2+}]$_i$ = 1.0 vs. 1.55 ± 0.12 at [Ca^{2+}]$_i$ < 10 nM, $n = 9$, $P < 0.05$). The spontaneous actin polymerization promoted by lowering [Ca^{2+}]$_i$ was not inhibited by addition of GDP-β-S, nor by pretreatment of the cells with pertussis toxin (Fig. 3), suggesting that this effect was not mediated by a GTP-binding protein. Under low calcium conditions ([Ca^{2+}]$_i$ < 10 nM), the receptor-mediated pathway for actin assembly remained functional, as evidenced by the response to the subsequent addition of fMLP, which resulted in further G- to F-actin transformation (Fig. 3). Thus, calcium does not appear to play a permissive role in chemoattractant-induced actin assembly.

Because reduction of [Ca^{2+}]$_i$ in the permeabilized cells led to an unexpected spontaneous actin polymerization, it was of interest to establish the effects of increasing [Ca^{2+}]$_i$ under otherwise comparable conditions. Electroporated cells were permeabilized in EGTA-buffered media containing physiologic free [Ca^{2+}] (100 nM). The appropriate amount of $CaCl_2$ (calculated using the algorithm of Fabiato and Fabiato, 1979) was then added to obtain the desired free [Ca^{2+}], and F-actin content was measured using NBD-phallacidin. The results of these experiments are summarized in Fig. 4. Increasing the medium free [Ca^{2+}], and therefore also [Ca^{2+}]$_i$, above the physiological level of 100 nM caused substantial actin disassembly. This effect was evident at levels of free [Ca^{2+}] above 500 nM and appeared to be maximum at ≥10 μM (Fig. 4 *a*). The calcium-induced actin disassembly was time dependent, peaking between 30 s and 1 min and returning to near baseline levels by 5 min (Fig. 4 *b*). Calcium-induced depolymerization was not mediated

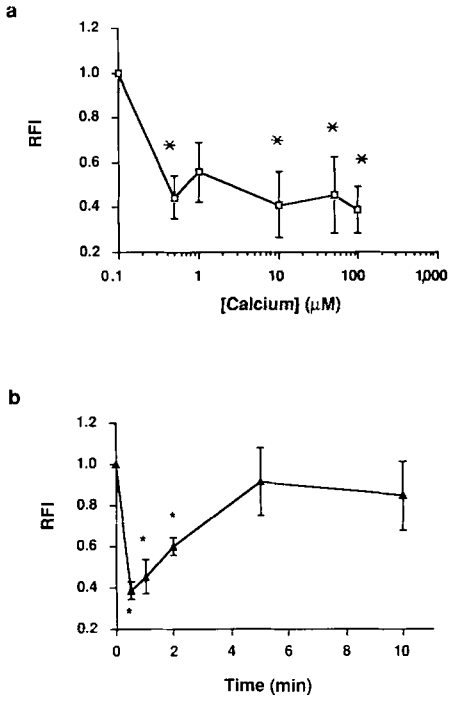

Figure 4. (*a*) Effect of varying free calcium on the F-actin content of electropermeabilized neutrophils. Cells were permeabilized in EGTA-buffered medium containing 100 nM free calcium (RFI = 1). After 1 min equilibration at 37°C, additional $CaCl_2$ was added to give the specified free calcium concentration (0.5, 1, 10, and 100 μM), calculated according to the method of Fabiato and Fabiato (1979). Each value represents the mean ± SEM of four determinations. Asterisks indicate $P < 0.05$ with respect to the control (100 nM free calcium), determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). (*b*) Time course of the changes in F-actin content induced by elevated intracellular calcium. Electropermeabilized neutrophils were equilibrated with EGTA-buffered medium containing 100 nM free calcium (RFI = 1). After 1 min, additional $CaCl_2$ was added to elevate the free calcium concentration to 10 μM and samples were taken at the time periods specified. Each value represents the mean ± SEM of five determinations. Asterisks indicate $P < 0.05$ with respect to the control (100 nM free calcium), determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).

by GTP-binding proteins, inasmuch as it was not inhibited by either the addition of 200 μM GDP-β-S to electropermeabilized cells (in six experiments, RFI with 10 μM Ca^{2+} was 0.60 $\pm$ 0.16 vs. 0.53 $\pm$ 0.28 in the absence and presence of GDP-β-S, respectively), or by pretreating intact cells with pertussis toxin before permeabilization (RFI in 10 μM Ca^{2+} was 0.59 $\pm$ 0.26, $n = 4$). These conditions are known to prevent fMLP-induced actin assembly, a GTP-protein-mediated event (Downey et al., 1989; Therrien and Naccache, 1989).

We compared the relative F-actin content of neutrophils as a function of $[Ca^{2+}]_i$ in the presence and absence of fMLP. The results are summarized in Fig. 5. Perusal of these data indicates that actin polymerization can be induced by reducing $[Ca^{2+}]_i$, as well as by treatment with fMLP at constant $[Ca^{2+}]_i$. Thus, the effect of the chemoattractant is equivalent to reducing the susceptibility of the filaments to disassembly by calcium. As the level of free $[Ca^{2+}]_i$ was increased above 1 μM, exposure to fMLP failed to produce significant polymerization.[2] At supraphysiological $[Ca^{2+}]_i$, actin disassembly resulted regardless of the order of exposure to high Ca^{2+} and fMLP. Moreover, raising free Ca^{2+} above 1 μM after stimulation with fMLP at normal $[Ca^{2+}]$ completely reversed the chemoattractant-induced actin assembly (in five experiments RFI for fMLP at 100 nM Ca^{2+} was 2.14 $\pm$ 0.24 versus 0.78 $\pm$ 0.16 for fMLP at 100 nM Ca^{2+} followed by 10 μM Ca^{2+}). Therefore, the depolymerizing effect of Ca^{2+} supersedes the polymerizing action of fMLP.

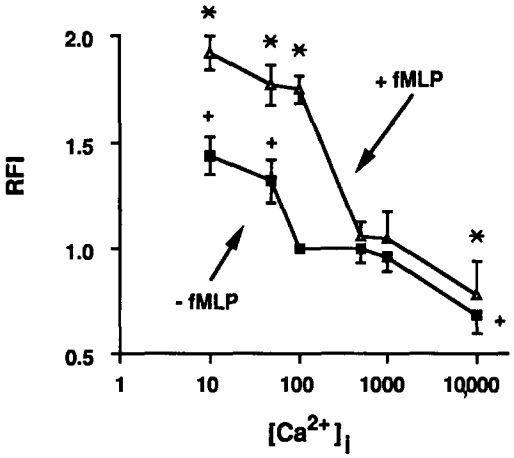

Figure 5. Effect of fMLP on the F-actin content of electropermeabilized neutrophils equilibrated with EGTA-buffered media of varying free calcium concentration. The cells were permeabilized in media of the specified free calcium concentration, preequilibrated for 1 min, and then incubated with (*top curve*) or without 10^{-8} M fMLP for an additional minute. Relative F-actin content was assayed by NBD-phallacidin staining. All data are expressed relative to the F-actin content of untreated cells suspended in 100 nM free calcium (RFI = 1). Each value represents the mean $\pm$ SEM of five determinations. (+, without fMLP) or (*, with fMLP) indicates $P < 0.05$ with respect to the appropriate control (100 nM free calcium), determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). Additionally, for $[Ca^{2+}]_i$ of <10, 50, and 100 nM, the values of F-actin content in the cells exposed to fMLP are significantly higher than the comparable cells in the absence of fMLP ($P < 0.05$).

Calcium Ionophore vs. Electroporation-induced Increases in $[Ca^{2+}]_i$

The effects on actin assembly were diametrically opposed when $[Ca^{2+}]_i$ was raised by electroporation when compared with the use of ionophores. The latter method induced a delayed polymerization (increased F-actin content), whereas increasing $[Ca^{2+}]_i$ in electroporated cells decreased F-actin content (cf. Figs. 1 and 4). The experiments described in this section were intended to reconcile these apparently discrepant observations. Treatment of intact cells with ionomycin in calcium containing solutions elicited a shape change associated with actin assembly (Fig. 6 *c*). As shown in Fig. 6 *a*, this response was partially inhibited by omission of extracellular calcium, suggesting that mobilization of internal calcium stores suffices to generate actin polymerization, or that the effects of the ionophore are unrelated to $[Ca^{2+}]_i$. It is unlikely that the response to ionomycin is nonspecific, due for instance to the insertion of the ionophore into the plasma membrane bilayer. This was concluded because, as described by others (Howard and Wang, 1987), addition of the structurally unrelated calcium ionophore A23187 also resulted in actin assembly (Fig. 6, *b* and *d*). As was the case for ionomycin, omission of external calcium decreased, but did not entirely eliminate the response to A23187 (Fig. 6 *b*).

On the other hand, experiments with pertussis toxin suggest that the response generated by the ionophores is not simply due to elevation of $[Ca^{2+}]_i$, which then acts as a second messenger, mimicking the release of calcium induced by inositol 1,4,5-trisphosphate (IP_3). The generation of IP_3 is attributed to activation of a phosphoinositide-specific phospholipase C by a pertussis toxin-sensitive GTP-binding protein (Cockcroft and Gomperts, 1985). Because the action of the GTP-binding protein precedes the liberation of IP_3 and the release of calcium from internal stores, treatment with pertussis toxin is not anticipated to affect the response to elevated $[Ca^{2+}]_i$. Contrary to this prediction, pertussis toxin prevented the polymerization stimulated by both ionomycin and A23187 (Fig. 6, *a* and *b*), as has been observed by others (Shefeyk et al., 1985). The observed inhibition, therefore, is suggestive of an effect of the calcium ionophores at a stage preceding the activation of GTP-proteins, likely involving surface receptors. Furthermore, addition of either A23187 or ionomycin in a high calcium medium (1 mM) to cells pretreated with pertussis toxin not only failed to induce the normal decrease in light scattering, but instead resulted in a scattering increase, corresponding to actin depolymerization (Fig. 6, *c* and *d*).[3]

It is conceivable that, because of the comparatively high

2. For these experiments, the cells were resuspended in the permeabilization medium containing the specified $[Ca^{2+}]$ and equilibrated for 1 min before exposure to fMLP for an additional minute. Unlike the cells in Fig. 4, the control cells for these experiments, which remained in the permeabilization medium for the same period of time (2 min), did not demonstrate significant disassembly induced by $[Ca^{2+}]_i$ in the range of 500 nM to 1 μM. Since the calcium-induced actin disassembly is time dependent (Fig. 4 *b*), it is possible that the cells had returned to their baseline at the time of fixation.

3. While this decrease in light scattering was observed in four out of five experiments, a decrease in F-actin content was observed in only three out of six experiments and the mean of the experiments was not significantly <1. We interpret this to reflect incomplete inhibition of the GTP-binding protein by pertussis toxin in some of the experiments. Alternatively, under these conditions a decrease in light scattering may reflect changes in other cellular properties, such as granularity.

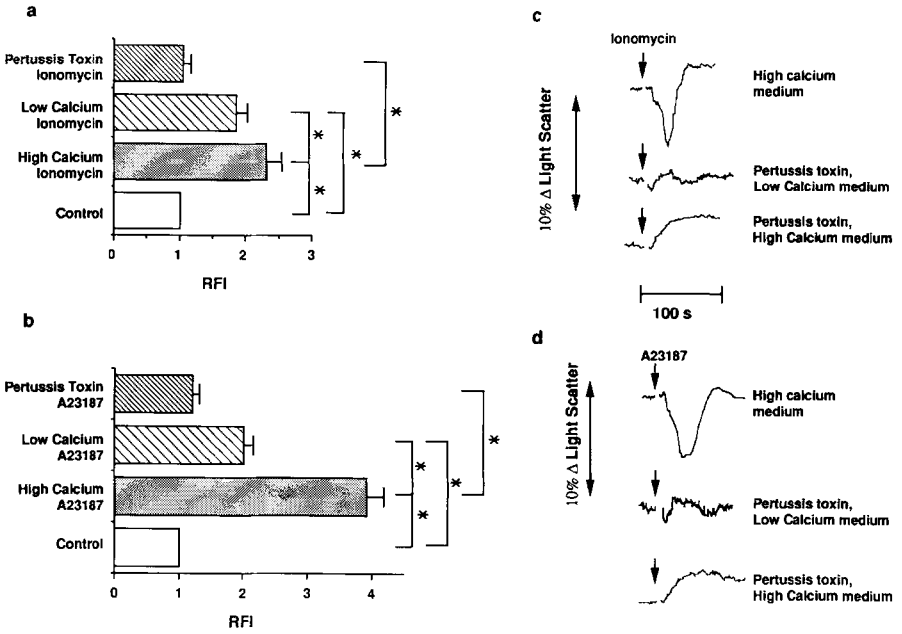

Figure 6. Effects of 10^{-6} M ionomycin (*a*) or 10^{-6} M A23187 (*b*) on the F-actin content of intact neutrophils. The cells were suspended in the presence or absence of the ionophores in medium with high (1 mM) or low (<10 nM) free calcium for 1 min. Where indicated, the cells treated with pertussis toxin (500 ng/ml for 120 min at 37°C) in high calcium medium before the addition of the ionophores. Data are expressed relative to the F-actin content of untreated cells. Each value represents the mean ± SEM of six determinations. Asterisks indicate $P < 0.05$ with respect to the control (100 nM free calcium), determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). Changes in right angle light scattering in response to exposure to ionomycin (*c*) or A23187 (*d*). Control cells were suspended in medium containing high (1 mM) calcium (*top traces*). The middle and bottom traces illustrate the response of cells pretreated with pertussis toxin as above and then exposed to either ionomycin or A23187 in low (<10 nM) or high (1 mM) calcium medium, as indicated. The arrow indicates the time at which the specified ionophore was added. Each trace is representative of at least four determinations. Note that in cells pretreated with pertussis toxin, the addition of either ionophore in high calcium medium results in an increase in light scattering.

concentration of extracellular calcium, a supraphysiological $[Ca^{2+}]_i$ is attained in the vicinity of the plasma membrane upon treatment with the ionophores. This abnormally high $[Ca^{2+}]_i$ could stimulate phospholipid hydrolysis by phospholipase A_2, a calcium-sensitive enzyme, generating arachidonic acid (Matsumoto et al., 1988). The products of lipid hydrolysis by phospholipase A_2 (e.g., arachidonic acid) or one of their metabolites, such as platelet activating factor (PAF) or leukotriene B_4 (LTB_4) (Naccache, 1987; Graff and Anderson, 1989), could be responsible for actin assembly, through a receptor-mediated (and in the case of PAF and LTB_4, pertussis toxin-sensitive) pathway. To investigate the possibility that PAF was signaling actin assembly, the neutrophils were pretreated with two chemically unrelated PAF antagonists: WEB-2086 and L659,989 (Hellewell and Williams, 1989). As demonstrated in Table I, these antagonists inhibited PAF-induced actin assembly but did not attenuate the responses induced by A23187 or ionomycin, making it unlikely that PAF was mediating the effects of the ionophores.

We next investigated whether formation of LTB_4 and subsequent interaction with its receptor could account for the polymerization of actin triggered by the ionophores. For this purpose, we used a specific LTB_4 antagonist,[4] compound LY-223982 (Shappell et al., 1989; Omann et al., 1989). As shown in Fig. 7, pretreatment of the cells for 1 min with this agent largely abolished the response to exogenously added LTB_4 (10^{-8} M). A similar treatment with LY-223982 completely inhibited actin polymerization induced by either A23187 or ionomycin,[5] but not that induced by fMLP (Fig. 7). The finding that LY-223982 had no effect on the response to fMLP indicates that the inhibitory effects of the leukotriene antagonist are specific. Furthermore, treatment with LY-223982 did not abolish the ionophore-induced increase in $[Ca^{2+}]_i$ (data not shown). Thus, the data suggest that the actin polymerization induced by either A23187 or ionomycin is likely secondary to ionophore-induced stimulation of phospholipase A_2 in response to the elevated $[Ca^{2+}]_i$, resulting in arachidonate release and oxidation to LTB_4. The leukotriene can then interact with plasma membrane receptors of the same or vicinal cells, stimulating actin polymerization through a pertussis toxin-sensitive GTP-binding protein. Such a mechanism would account for two earlier observations: (*a*) that the actin-polymerizing effect of the ionophores is sensitive to pertussis toxin and (*b*) that there is a slight delay between the ionophore-induced calcium influx and the initiation of actin polymerization.

In conclusion, several lines of evidence strongly suggest that fMLP-induced actin polymerization does not depend on an increase of $[Ca^{2+}]_i$. First, significant actin assembly in response to the chemotactic peptide was noted (*a*) in cells pretreated with ionomycin in low calcium medium, under conditions where fMLP had little effect on $[Ca^{2+}]_i$ (Fig. 1), and (*b*) in electropermeabilized cells equilibrated with media containing high concentrations of calcium buffering agents (Fig. 2). Though the occurrence of small, localized changes

4. LY-223982 is a specific LTB_4 receptor antagonist with no other known actions (Shappell et al., 1989; Omann et al., 1989). In preliminary studies (Lue, D., and S. Grinstein, unpublished observations) we have demonstrated that LY-223982 inhibits LTB_4-induced superoxide production but not that due to either fMLP or arachidonic acid, supporting its specificity of action on the LTB_4 receptor.

5. A similar dose of LY-223982 inhibited actin polymerization more thoroughly in ionophore-stimulated cells than in LTB_4-treated ones, despite the fact that the ionophore response was larger. This may be due to the unmasking of the direct actin depolymerizing effect of elevated $[Ca^{2+}]_i$ depicted in Fig. 4.

Table I. Effect of PAF Antagonists on Calcium Ionophore-induced Actin Assembly

Condition	RFI
Control	1.0
PAF 10^{-6} M	2.30 ± 0.16
PAF 10^{-6} M (PreRx WEB 2086)	1.37 ± 0.13
PAF 10^{-6} M (PreRx L659,989)	1.14 ± 0.16
A23187 10^{-6} M	3.47 ± 0.32
A23187 10^{-6} M (PreRx WEB 2086)	3.18 ± 0.41
A23187 10^{-6} M (PreRx L659,989)	3.03 ± 0.15
Ionomycin 10^{-6} M	3.56 ± 0.27
Ionomycin 10^{-6} M (PreRx WEB 2086)	3.48 ± 0.29
Ionomycin 10^{-6} M (PreRx L659,989)	2.80 ± 0.13

Where indicated, intact neutrophils were pretreated with either WEB 2086 or L659,989 at a concentration of 10^{-5} M for 10 min at 37°C prior to stimulation with PAF or the indicated ionophore. The reaction was then terminated and actin polymerization determined using NBD-phallacidin as described in Materials and Methods. Data are presented as RFI ± SEM of $n = 5$ experiments.

in $[Ca^{2+}]_i$ could not be rigorously excluded, additional evidence makes it unlikely that an increase in cytosolic Ca^{2+} initiated the conversion of G- to F-actin. Increasing $[Ca^{2+}]_i$ in electropermeabilized cells, in the absence of ionophores or other neutrophil stimuli, resulted in actin disassembly (Fig. 4). Such an actin-depolymerizing effect of calcium has been postulated in other cell types such as adrenal chromaffin cells (Sontag et al., 1988; Burgoyne et al., 1989). Furthermore, the prevailing $[Ca^{2+}]_i$ appeared to determine the sensitivity of the actin assembly pathway to subsequent stimulation with fMLP, with progressively smaller responses obtained at higher $[Ca^{2+}]_i$ (Fig. 5). Finally, we reconciled the disparate effects of the calcium ionophores and of electropermeabilization on the induction of actin assembly. Increased F-actin content in response to ionophores is sensitive to pertussis toxin, implying mediation by GTP-binding proteins, and is inhibited in the presence of an LTB_4 antagonist. These findings suggest that the ionophores activate phospholipase A_2, and that the arachidonate released is metabolized via the lipoxygenase pathway. According to this model, LTB_4 would be directly responsible for activation of the cells. In contrast, equilibration of permeabilized cells in high $[Ca^{2+}]_i$ media exposes a direct actin depolymerizing effect of calcium (Fig. 4). The latter can be unmasked in intact cells treated with ionophore, provided the G proteins that mediate stimulation of actin assembly are inhibited by pertussis toxin (Fig. 6, *c* and *d*).

We therefore believe that, under physiological conditions, the result of increased $[Ca^{2+}]_i$ during stimulation is to promote actin disassembly, an effect that may be mediated through calcium sensitive actin-serving protein(s) such as gelsolin, which is known to be activated by $[Ca^{2+}]_i$ in the range of 500–1,000 nM (Yin et al., 1980). Thus, in response to a receptor-mediated stimulus such as fMLP, actin polymerization is initiated by a pathway involving a GTP-binding protein, perhaps via the PIP_2-induced dissociation of profilin from the profilactin complex (Lassing and Lindberg, 1985; Stossel, 1989). This polymerization pathway would be independent of changes in $[Ca^{2+}]_i$ and thus compatible with our observations. Simultaneously or shortly thereafter, the transient increase in $[Ca^{2+}]_i$ would result in activation of calcium-sensitive actin-severing protein(s), promoting actin depolymerization. The ensuing sequential assembly and disassembly of actin filaments could be essential for neutrophil function. In this regard, oscillations in both $[Ca^{2+}]_i$ (Marks and Maxfield, 1990) and F-actin content (Omann et al., 1989) were recently reported to occur in stimulated phagocytes. Such oscillations may be an indication that a series of actin assembly/disassembly steps, rather than a single cycle, is required for chemotaxis and/or phagocytosis.

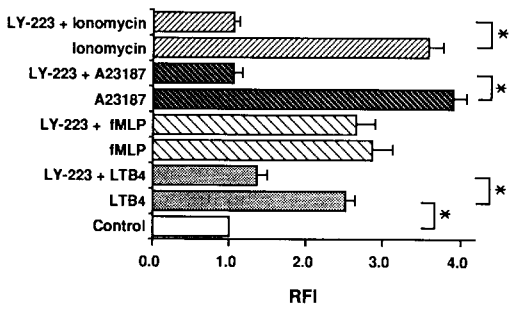

Figure 7. Effects of the leukotriene antagonist LY-223982 on the F-actin content of intact neutrophils exposed to 10^{-8} M LTB_4 ($n = 9$), 10^{-8} M fMLP ($n = 5$), 10^{-6} M A23187 ($n = 7$), or 10^{-6} M ionomycin ($n = 7$) for 1 min at 37°C as assayed by NBD-phallacidin staining. Where indicated, LY-223982 (10^{-5} M) was added 1 min before stimulation. Asterisks indicate significant ($P < 0.05$) differences between the indicated samples. Each value represents the mean ± SEM of the number of determinations.

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